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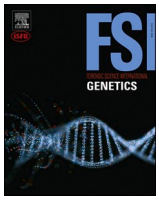
DOI: https://doi.org/10.1016/j.fsigen.2021.102524

Forensic Science International: Genetics 53 (2021) 102524

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsigen



Degradation of human mRNA transcripts over time as an indicator of the time since deposition (TsD) in biological crime scene traces

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ARTICLE INFO

Keywords:
Forensics
Body fluids
Transcriptome analysis
RNA degradation
Time since deposition (TsD)

ABSTRACT

Knowledge about the age of a stain, also termed as time since deposition (TsD), would provide law-enforcing authorities with valuable information for the prosecution of criminal offenses. Yet, there is no reliable method for the inference / assessment of TsD available. The aim of this study was to gain further insight into the RNA degradation pattern of forensically relevant body fluids and to find candidate markers for TsD estimation. Blood, menstrual blood, saliva, semen and vaginal secretion samples were exposed to indoor (dark, room temperature) and outdoor (exposed to sun, wind, etc. but protected from rain) conditions for up to 1.5 years. Based on expression and degradation analyses, we were able to identify body fluid specific signatures and RNA degradation patterns. The indoor samples showed a marked drop in RNA integrity after 6 months, while the outdoor samples were difficult to interpret and therefore excluded for some of the analyses. Up to 4 weeks, indoor samples showed more stable and less degrading transcripts than outdoor samples. Stable transcripts tended to be significantly shorter than degrading ones or transcripts, which are neither degrading nor stable. We reinforced the body fluid specific and the housekeeping gene nature of previously reported markers. With an unbiased approach, we selected stable and degrading genes for each body fluid in the short term and assessed their integrity during extended storage. We identified several stable and degrading gene transcripts, which could be tested in a targeted assay to assess the TsD interval e.g. by analyzing the ratio of degrading vs stable transcripts. In conclusion, we were able to detect RNA degradation patterns in samples being aged up to 1.5 years and identified several candidate markers, which could be evaluated for TsD estimation.

1. Introduction

DNA profiling is routinely used in forensic genetics to identify the person from whom a biological sample was derived [1]. In recent years, mRNA profiling has emerged as a new method for the identification of body fluids and tissues. Since differentiated tissues possess a unique transcriptome, RNA transcripts restricted in expression can be used to identify body fluids, tissues or organ types [2–21]. Although RNA is prone to fast *ex-vivo* degradation due to its chemical structure, RNases present in the environment, humidity and UV-radiation, mRNA has proven to be more stable than expected in dried body fluid stains, depending on the storage conditions [22–24]. For example mRNA transcripts were successfully detected in samples up to 23 years old [22].

However, there is no reliable method available for determining the age of biological traces, also known as time since deposition (TsD). Accurate estimation of TsD would not only allow the verification of witnesses' statements or alibis; it would also help to identify the true relevance of a trace for the investigative process by linking its deposition to the time point of the crime [25]. In the past, spectroscopy, chromatography or electron spin resonance have been used to estimate the age of blood stains, mostly based on the time-dependent change of color [25–28]. Since these methods are limited to blood stains, traces such as saliva, semen or vaginal secretion cannot be analyzed likewise.

Another approach to determine TsD is the time-dependent degradation of biomolecules such as DNA or RNA. As a possible indicator of the age of bloodstains, Bauer et al. suggested to quantify the degradation of RNA by semi-quantitative duplex and competitive RT-PCR, that compared the relative abundance of two housekeeping gene transcripts (β-actin and cyclophilin) and an external standard [29]. Anderson et al. used quantitative PCR (qPCR) to estimate the age of bloodstains based

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https://doi.org/10.1016/j.fsigen.2021.102524
Received 29 December 2020; Received in revised form 8 April 2021; Accepted 26 April 2021
Available online 4 May 2021

on the relative degradation of β-actin messenger RNA (mRNA) and 18S-ribosomal RNA (rRNA) amplicons over a time course of 150 days [30]. The ratio of the two RNA species changed in a linear fashion over time. Hampson et al. used a similar approach for plucked hair, aged naturally for up to 3 months [31]. The relative mRNA / rRNA expression versus the age of the stain followed a linear trend for up to 60 days. Besides, Qi et al. examined the β-actin / 18S-rRNA ratio in blood, aged for 28 days, from 16 Han Chinese [32]. The ratio of the two RNA species changed in a linear fashion but a gender-effect was observed. In a follow up study, Anderson et al. proposed an improved method to estimate the age of blood stains by analyzing two different amplicon sizes within 18S-rRNA and β-actin mRNA [33]. The large amplicons disappeared more rapidly than the small ones and 18S-rRNA was more stable than β-actin mRNA. By using a multivariate analysis approach where results of several amplicon pairs were combined, an adequate age estimation was possible. Based on a literature search, Alshehhi et al. selected mRNA, miRNA and reference markers to examine the relative expression ratio in blood, saliva and semen samples using a RT-qPCR approach [34,35]. mRNA markers showed a unique degradation pattern, while miRNA markers and U6 (reference gene) were highly stable. Analysis of the relative expression ratios (more / less stable RNA markers or body fluid specific markers / reference genes) were proposed as possible indicator of TsD.

The introduction of massively parallel sequencing (MPS) has revolutionized and facilitated RNA analyses. Lin et al. successfully sequenced the transcriptomes of freshly collected body fluids (oral mucosa / saliva (buccal), circulatory blood, menstrual blood and vaginal secretion) and aged stains (up to 6 weeks) [36]. Most of the sequences (85%) were of high quality (average sequence phred score above Q30). They assessed the global gene expression levels during ageing of samples and characterized the differential RNA degradation within the body fluids. Weinbrecht et al. sequenced the transcriptome of blood, saliva, semen, and vaginal secretion samples, aged up to 1 year [37]. The degradation patterns of the transcripts in each samples type was monitored to better understand the aging of forensically relevant body fluids. Transcript abundance declined in the different body fluids over time. The greater the transcript abundance at time point 0, the longer a transcript could be detected in the respective stains. Saliva samples had to be excluded from the study, since transcript abundance decreased drastically and uneven over time.

In our previous study we evaluated several RNA-Seq workflows to analyze fresh and aged body fluid samples [38]. We found that phenol / chloroform and rRNA-depletion had an adverse effect on library preparation, sequencing and the downstream analyses of both, human and bacterial RNA. In general, aged samples showed a higher level of RNA degradation than fresh ones and a lower bacterial diversity. Besides and as expected, we identified source-specific signatures from human and microbial RNA. The aim of the present study was to gain further insight into the RNA degradation pattern of forensically relevant body fluids and, ideally, to identify markers for TsD estimation. We applied the optimized workflow to body fluid samples that were aged under indoor and outdoor conditions for up to 1.5 years. In a parallel manuscript, we will assess the time dependent changes in the microbial composition of biological crime scene traces using microbial RNA markers.

2. Material and methods

2.1. Body fluid sampling

Forensically relevant body fluids (blood, menstrual blood, saliva, semen, vaginal secretion) were collected on sterile cotton swabs (Milian, Nesselnbach, Switzerland). Menstrual blood and vaginal secretion were self-collected on swabs by the donors. Semen and saliva were self-collected in sterile tubes and 50 μl each were spotted on the swabs. Blood was obtained by venipuncture and collected in EDTA coated tubes, and 50 μl was directly pipetted onto each swab. The body fluid samples, collected from 3 different individuals each, were exposed to two different environmental conditions: 1) Indoor samples were stored at room temperature in a dark and dry place; 2) Outdoor samples were placed on a flat rooftop, exposed to sun, wind, etc. but protected from rain. The fresh samples (deposition / day 0) were analyzed immediately after sample collection. The aged samples were analyzed after 1 day, 7 days, 4 weeks, 6 months, 1 year and 1.5 years. In total 210 samples were analyzed: 5 body fluids, 3 donors, 7 time points, 2 environmental conditions. The sampling was approved by the local ethics commission (declaration of no objection KEK-No. 24-2015).

2.2. RNA extraction

RNA was extracted using the ReliaPrep™ RNA Cell Miniprep kit (Promega, Dübendorf, Switzerland). We used the protocol for $>5 \times 10^5$ to 2×10^6 cells according to the manufacturer's instructions, with the following modifications: Stains were incubated in BL+TG buffer for 2–3 h and the final elution volume was 30 μl. RNA quantity was assessed using the QuantiFluor® RNA HS System (Promega) as described by the manufacturer.

2.3. Library preparation and sequencing

We applied an optimized RNA-Seq workflow, which we had tested on body fluid samples and described in our previous publication [38]. Total RNA libraries were constructed using the Trio RNA-Seq kit (Nugen, Leek, The Netherlands) according to the manufacture's protocol, without ribosomal RNA depletion. The kit is optimized for low input and challenging samples and is suitable to generate sequencing libraries from total RNA using universal priming [39] and is layed-out for 500 pg to 50 ng total RNA. If 50 ng in 10 μl could not be reached, the maximal volume of RNA extract (10 μl) was used. Most samples (97%) reached the recommended input amounts. Libraries were checked for quality with the TapeStation 4200 system for nucleic acid analysis (Agilent Technologies, Santa Clara, USA). Libraries generated from fresh (day 0), 1 day, 7 days and 4 weeks old stains were sequenced on the Illumina HiSeq4000 platform, generating 1×125 bp reads using a single indexing (8 bp) strategy. Since the Illumina HiSeq4000 platform at our sequencing facility (Functional Genomics Center Zurich, FGCZ) was replaced during the course of this project, the 6 months, 1 and 1.5 year old samples were sequenced on an Illumina NovaSeq6000 platform, generating 1×100 bp reads using a unique dual indexing strategy.

2.4. RNA-Seq data analysis

Reads were quality-checked using FastQC. Sequencing adapters were removed with Trimmomatic [40]. Putative mRNAs were separated from rRNAs using SortMeRNA [41]. Reads $\geq$ 20 bases and with an overall average phred quality score $\geq$ 10 were aligned to the reference genome and transcriptome of Homo sapiens (FASTA and GTF files, respectively, downloaded from GRCh38, no patches) with STAR v2.5.1 [42].

Based on the aligned reads, the relative expression levels of the transcripts have been quantified for each sample using the R package *GenomicRanges* [43]. Minimum mapping quality, as well as minimum feature overlaps was set to 10. Multi-overlap was allowed. The trimmed means of M-values (TMM) normalization from the R package *edgeR* was applied [44], which is a simple and effective method for estimating relative RNA expression levels from RNA-Seq data. The integrity of the transcripts was calculated using the transcript integrity number (TIN) [45]. This calculation assigns a score ranging from 0 to 100 to each transcript, where 0 indicates that no fraction of the transcript could be reconstructed by the sequencing reads and 100 represents a fully intact transcript. Once every transcript had an associated TIN number, the mean over those values was assigned to be the TIN number of the respective gene.

The expression and degradation profile for each sample is

approximated by using the TMM-normalized counts and TIN values, respectively. To better understand how such metrics define the samples, we performed a Non-metric Multi-Dimensional Scaling (NMDS) analysis for the 100 genes with the highest overall variance in the cohort. NMDS attempts to represent the pairwise dissimilarity between objects in a low-dimensional space and substitutes the original distance data with ranks, which generally is a more robust approach for data, which do not have an identifiable distribution. Only genes with at least 10 observations in at least half of the samples in the cohort have been selected for the NMDS analysis. Indoor and outdoor samples were separated.

With the help of the TIN numbers we searched for stable and degrading gene transcripts in samples of up to one month of age, for which more time points were analyzed per time interval. For each time point, the mean across the three replicates (donors) was taken. The following criteria determined the transcript trend: 1) *degrading*: TIN values were interpolated over time using the *poly*() and *lm*() functions from the R package stats. The interpolation coefficients are those resulting from the linear regression. Thresholds: maximum TIN at day 0 (deposition); minimum TIN at day 30 (4 weeks); interpolation $R^2 > 0.5$; R^2 interpolation p-value <0.05; interpolation coefficient <-1. 2) *stable*: the dispersion coefficient for a gene is defined as the standard deviation of the TIN values divided by their mean over the time points. Threshold: dispersion coefficient over time <0.05. Any gene not falling in the two categories above is referred to as having a "NA" trend. For degrading transcripts, the level of degradation was calculated as the relative decrease (in %) between day 0 (deposition) and day 30 (4 weeks), i.e. (TIN0-TIN30)/TIN0 * 100. Transcript length was divided into small (< 2k bp), medium (2k - 10k bp) or large (> 10k bp).

We performed an unbiased screening of the degradation patterns in order to identify putative markers showing a long term stability or a continuous degradation over time. To search for stable and degrading transcripts in samples up to 6 months of age the following criteria were applied: *1) stable gene transcripts:* dispersion coefficients below the 5-th percentile; *2) degrading gene transcripts*: interpolation coefficient above the 95-th percentile over the short term (up to 4 weeks) and a monotonic decreasing trend throughout the time course (up to 1.5 years).

2.5. Statistical methods

We employed the one-sided Wilcoxon rank sum test to compare the distribution of the TIN values in different groups, adopting Bonferroni-corrected significance thresholds depending on the number of tests performed.

3. Results

3.1. Sequencing

All except 2 of the 210 sequenced samples reached the targeted 40–50 million reads per sample. A fresh semen sample and a 6 months old vaginal secretion sample (indoor condition) showed 6.98 million reads and 17.8 million reads, respectively.

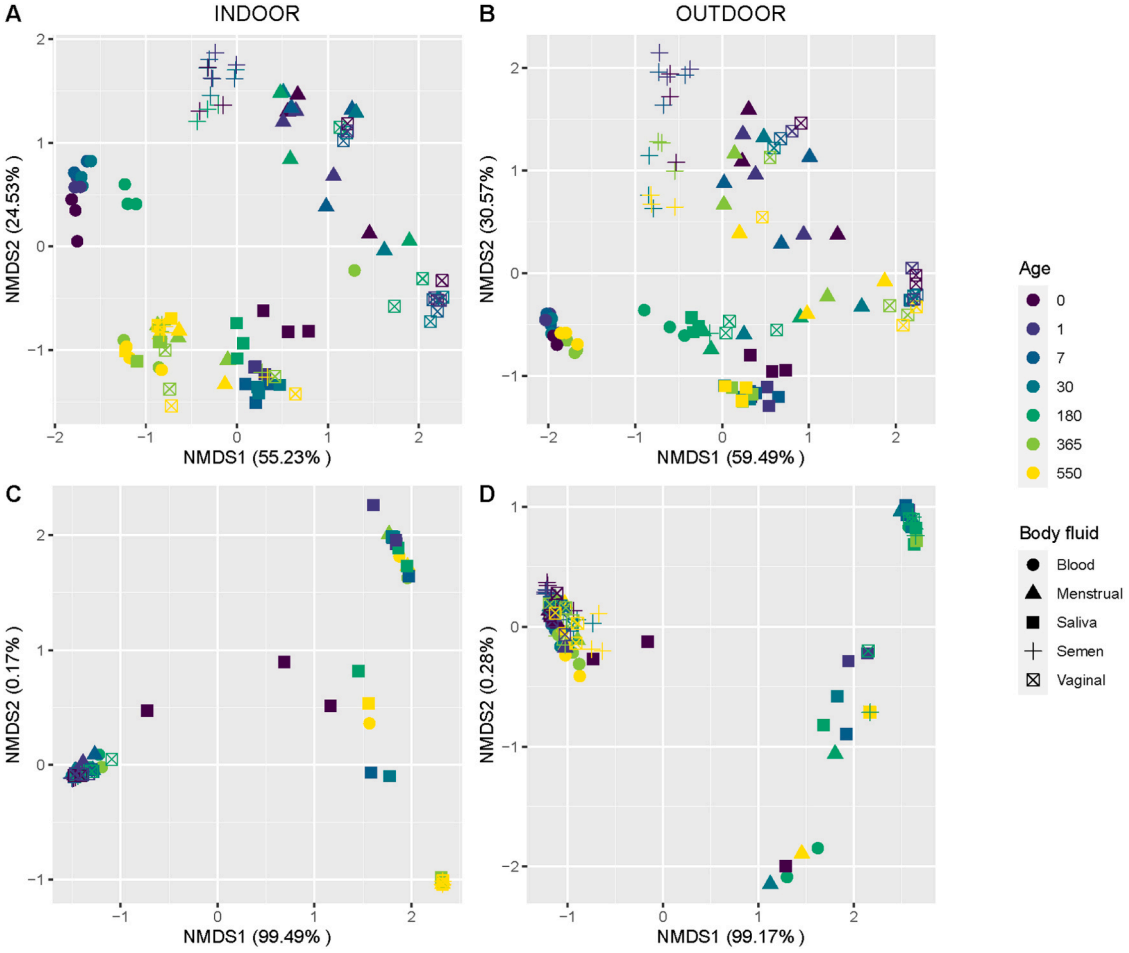

Fig. 1. NMDS-plots of the indoor (A) and outdoor (B) samples based on the expression values of the top-ranked 100 genes according to the expression variance across the samples. NMDS-plots of the indoor (C) and outdoor (D) samples based on the TIN values of the top-ranked 100 genes according to the TIN variance across the samples. *(For interpretation of the references to color in this figure, the reader is referred to the online version of this article.)*

3.2. Sample clustering based on expression and degradation profiles

NMDS-plots based on the gene expression profiles (Fig. 1A (indoor), 1B (outdoor)) revealed that the main separation (first NMDS components) is driven by the body fluids, which accounted for around 55% and 59% of the variance in indoor and outdoor samples, respectively. In particular, blood and semen were clearly separated, while some overlap of vaginal secretion and menstrual blood samples was visible. This can be expected as both originate from the urogenital tract. Saliva samples cluster together, but an overlap with other (non-saliva) body fluid samples was visible in the indoor samples. However, around 25% of the variance (second NMDS components) was still attributable to the time since deposition / age of the sample, more pronounced in the indoor samples.

NMDS-plots based on the TIN values for the indoor samples clearly separated time points up to 6 months from time points older than 6 months (Fig. 1C). In the indoor samples, it appears that most of the observed variation in degradation was due to age, as indicated by the separation along the first NMDS component which explained 99.5% of the variation. In contrast, NMDS-based clustering for the outdoor samples did not show a clear separation of the samples based on their age (Fig. 1D). In the outdoor samples, the quality of the samples seems to be the main factor for variation in degradation. This can be seen as a separation between the low quality samples (6 months and saliva) and the remaining samples along the first component, which explained 99% of the variation. Saliva samples did not cluster based on age, neither indoor nor outdoor.

3.3. Overall TIN distributions (up to 1.5 years)

In a next step, we analyzed the distributions of the calculated TIN values over time for each body fluid. Results are stratified by body fluid and condition (Fig. 2).

In general, TIN values decreased over time for blood, semen, menstrual blood and vaginal secretion. In blood, the TIN numbers were stable over 30 days and then began to drop. Saliva showed a unique TIN pattern. Already at deposition time, very low TIN values for mRNA in the indoor and outdoor condition were obtained. This finding was most likely due to the exceptionally low human read counts found for this body fluid. Therefore, TIN calculations were not regarded as reliable and saliva was excluded from further analyses.

A feature common to all indoor samples was a marked drop in mRNA TIN numbers after one year of exposure, indicating a high level of transcript degradation after this time point. In contrast, outdoor samples showed a more fluctuating pattern. Common to all 6 months old outdoor samples is a marked drop in TIN values to almost zero (indicating very degraded samples). We realized that for these samples the rRNA read counts were exceptionally high and might have been preferentially amplified.

3.4. Short term degradation patterns (up to one month)

We explored the short term degradation patterns in samples aged up to one month, for which more time points were analyzed per time interval. Based on the trends (see Materials and Methods) the transcripts were grouped into stable and degrading, as well as transcripts with no specific trend. Stable and degrading transcritps were observed in all body fluids and for both environmental conditions. The majority of the transcripts (77.58–99.72%) did not show a clear trend (referred to as "NA"). Indoor samples showed overall a higher number of stable gene transcripts (9.74–21.09%) and a lower number of degrading transcripts (0.27–7.17%), compared to outdoor samples (0.19–10.66% and 2.11–15.52%, respectively).

3.5. Relationship between gene features and degradation patterns

Another aspect investigated is whether specific characteristics such as GC-content, transcript length or the number of isoforms (splicing complexity) of a transcript can be associated with its tendency to show a stable or degrading pattern rather than no clear trend ("NA"). We included for each body fluid only those transcripts, which passed our expression threshold (see Materials and Methods). In addition, we stratified by transcript length (small $<$ 2k bp, medium 2k - 10k bp, large $>$ 10k bp). The results for all features per body fluid and conditions are shown in Supplementary Figs. 1–4. First, the GC-content was different between transcripts without a specific pattern ("NA") and those, which were degrading or stable (Supplementary Figs. 1–4, left panels). The GC-content in degrading transcripts was always higher than in stable gene

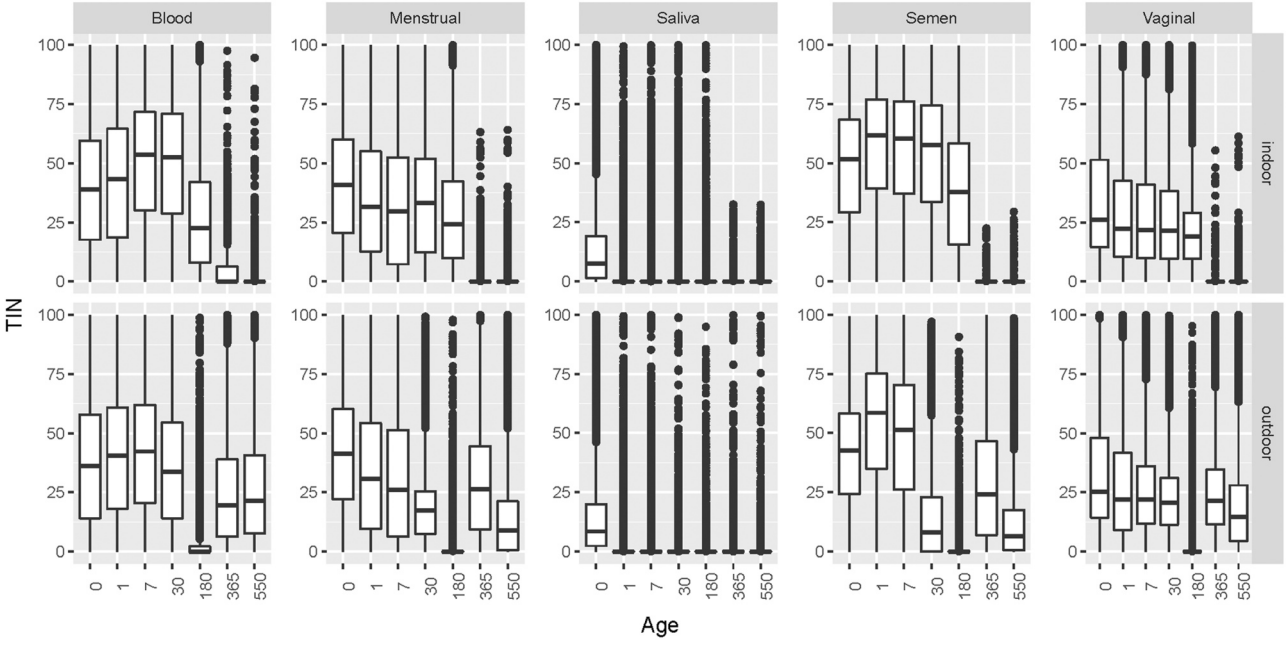

Fig. 2. Box-and-whisker plots of the TIN values (y-axes) in the different body fluids over time (days, x-axes). For each body fluid the data are stratified by the exposure condition (rows: "indoor", "outdoor").

transcripts, whereas depending on the body fluid, it was either lower or higher in "NA" transcripts. Second, stable transcripts tended to be significantly shorter than the degrading or "NA" genes, especially in the transcript groups of small or medium length (Supplementary Figures 1–4, middle panels). This suggests that larger transcripts are specifically prone to degradation. Third, splicing complexity showed few differences between degrading, stable and "NA" genes (Supplementary Figs. 1–4, right panels). In some cases, degrading transcripts showed a larger number of isoforms.

3.6. Commonality and uniqueness of transcript trends across body fluids

We then looked at whether some transcripts are stable / degrading in all body fluids or show a body fluid-specific trend. The majority of transcripts showed a time trend (stable or degrading) only in one body fluid (Fig. 3). In particular, the only transcripts showing a trend common to all body fluids were stable and from the indoor samples. No or few degrading transcripts were common to all 4 body fluids. In addition, vaginal secretion and menstrual blood samples tended to have more (and unique) degrading transcripts than other body fluids, while semen showed a large number of unique stable transcripts.

3.7. Different trends among indoor and outdoor samples

We also inspected cross-condition transcripts trends to see whether there are transcripts, showing the same trend under both indoor and outdoor conditions. Blood and vaginal secretion samples presented a relatively large number of transcripts, which are stable in both indoor and outdoor conditions (Fig. 4). Semen and menstrual blood samples showed very few stable transcripts common to both conditions, indicating a tendency to be more strongly affected by environmental exposure. The ratio of indoor-to-outdoor stable gene transcripts ranges from 120/1 in the semen samples, to 1/1 in the blood samples. On the other hand, differences between outdoor and indoor samples are much smaller for the degrading transcripts, with blood samples showing the highest indoor-to-outdoor ratio (7.86/1).

To estimate not only how many transcripts are degraded, but also to quantify the levels of decay, we analyzed the degrading genes in greater depth. A metric of high relevance is the transcripts fraction, which is lost at a given point in time. Indoor samples all displayed similar levels of degradation, whereas outdoor menstrual blood and semen samples displayed a significantly larger amount of very high-to-completely degraded transcripts (Supplementary Fig. 5). Consequently, the menstrual blood and semen samples again seemed to have suffered more from environmental exposure than the other samples.

3.8. Previously reported mRNA markers

In recent years, several body fluid specific mRNA markers have been identified and reported to be stable for a long period of time. Based on a literature search, we focused on two groups of genes: 1) genes specifically associated to a body fluid (Supplementary Table 1) and 2) housekeeping genes, i.e. genes putatively stable across body fluids (Supplementary Table 2).

Seventeen out of the selected 29 body-fluid specific transcripts (58%) fulfilled either the degradation or the stability criteria in our analysis (Fig. 5A). More importantly, all but two (*HBB* and *MMP3*) showed a trend only in one body fluid, strengthening the idea that those transcripts are indeed body fluid specific. In addition, 6 of those 17 gene transcripts showed a degrading pattern outdoor (*ANK1, CD93, FAM83D, MMP7, MUC4, PRM2*), one indoor (*ALAS2*) and one under both conditions (*MMP11*).

In addition, 9 of the 10 selected housekeeping genes were stable in stains stored indoors, with 5 of them showing this trend in all body fluids (*ACTB, B2M, GAPDH, UBC* and *UBE2D3*) and only 2 being body fluid specific (*UBE2S* in semen; *UBE2N* in vaginal secretion) (Fig. 5B). Only in the case of outdoor vaginal secretion samples, 3 transcripts (*GAPDH, UBE2D2* and *UBE2K*) were degrading.

The long term behavior of these selected transcripts was studied for the indoor samples only, since only few stable transcripts were detected in outdoor samples. For the body fluid specific gene transcripts, we found that, in general, they were stable up to 6 months (Supplementary Fig. 6). The exceptions were menstrual blood-specific transcripts, which showed visible degradation already after 30 days. The only two body fluid specific transcripts showing a degrading pattern (*ALAS2* and *MMP11* in menstrual blood) were completely degraded after 1 year. Similarly, the housekeeping genes were either largely degraded (blood and menstrual blood) or completely degraded (semen and vaginal secretion) after 1 year. Altogether, these results reinforce the body fluid specific and the housekeeping gene nature of previously reported

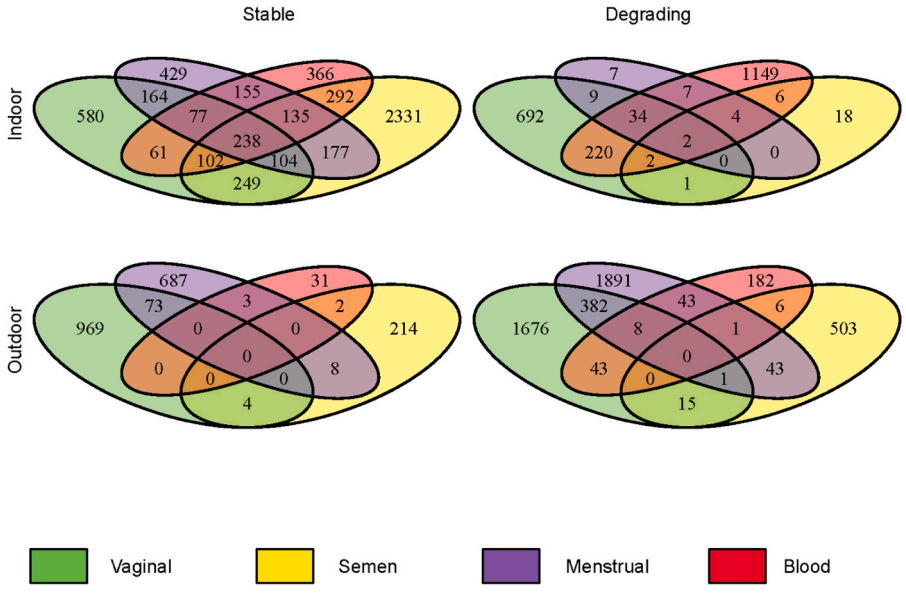

Fig. 3. Venn diagram displaying the number of transcripts showing a specific trend exclusive to one body fluid or shared by 2, 3 or 4 body fluids. The data are stratified by trend ("degrading", "stable") and exposure condition ("indoor", "outdoor"). *(For interpretation of the references to color in this figure, the reader is referred to the online version of this article.)*

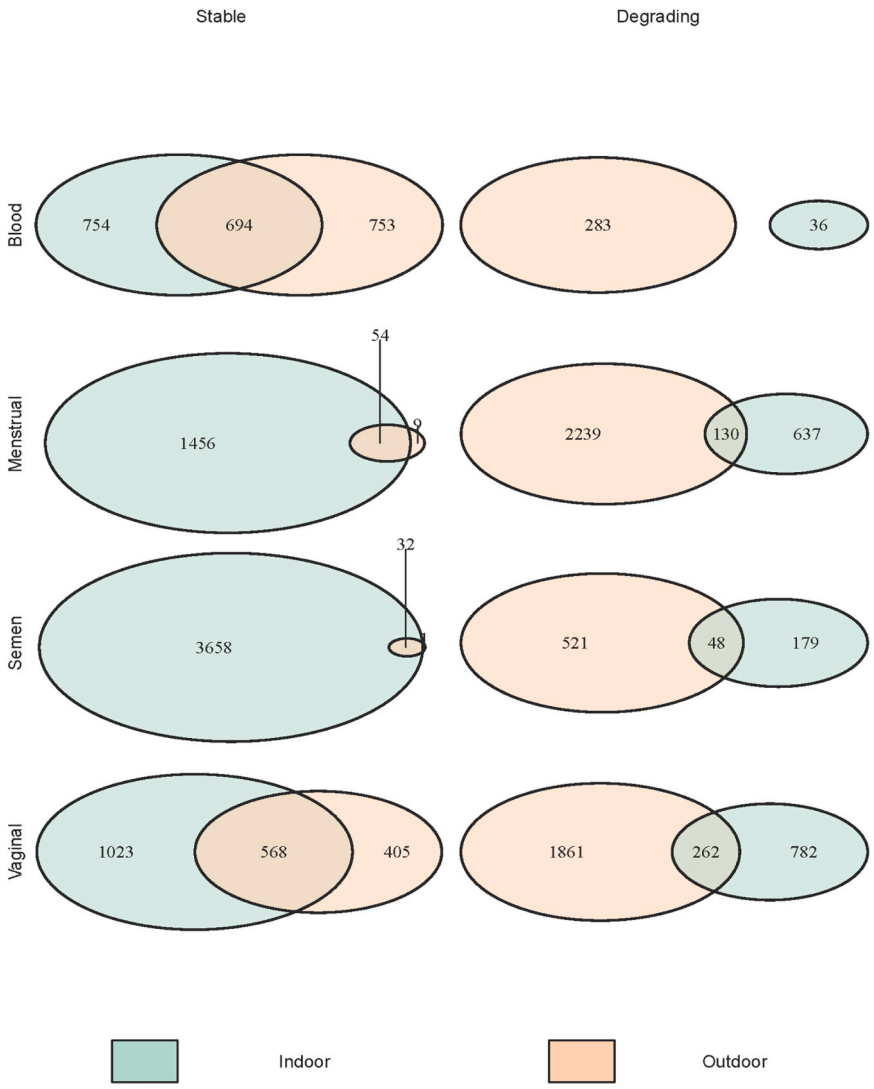

Fig. 4. Venn diagram showing the overlaps between transcripts with a specific trend (stable or degrading) in the indoor and outdoor samples. *(For interpretation of the references to color in this figure, the reader is referred to the online version of this article.)*

markers. However, no marker exhibited a significant resilience to degradation past 1 year of age.

3.9. Potential TsD mRNA markers

Finally, we performed an unbiased screening of the degradation patterns in order to identify putative markers showing a long term stability or a continuous degradation over time (Fig. 6). We performed this analysis only on indoor samples, since only few stable genes were detected in outdoor samples. An important observation was that after 1 year all transcripts seemed to be heavily degraded, while only a handful (mostly in blood), did not degrade by at least 50% after 1 year. The majority (10 out of 13) of the stable transcripts are of mitochondrial origin, while the remaining 3 are the alpha- and beta-hemoglobins (*HBA1, HBA2* and *HBB*). *HBA* and *HBB* are both well-known blood-specific genes.

Based on our observations so far, it seems evident that time points beyond 6 months are very hard to evaluate. Therefore, we only included samples up to 6 months of age for the calculation of the dispersion coefficient. A total of 91 transcripts were identified to be stable,(dispersion coefficients below the 5-th percentile throughout the 6 months), 24 for blood, 14 for menstrual blood, 69 for semen, 22 for vaginal secretion (the full list is provided in Supplementary Table 3). Some transcripts were stable in more than one body fluid.

In order to focus on the genes with the most robust degrading trends, we selected for each body fluid those transcripts with 1) an interpolation coefficient above the 95-th percentile over the short term (up to 4 weeks) and 2) a monotonic decreasing trend throughout the time course (up to 1.5 years). In total 53 transcripts fulfilled these criteria, 22 for menstrual blood, 8 for semen, 23 for vaginal secretion (the full list is provided in Supplementary Table 4). In all cases, complete degradation was observed after one year. According to these criteria, no potential degrading marker was found in blood.

4. Discussion

A reliable estimation of the TsD interval would aid law-enforcing authorities in the prosecution of criminal offenses. We set out to characterize the degradation pattern of human mRNA within body fluid stains stored under different conditions in order to characterize time-dependent changes. We assessed the stability of body fluid specific and housekeeping gene transcripts in those stains and identified potential markers to estimate the time since deposition of biological stains.

When assessing the gene expression profile using NMDS, we found that more than half of the variance within the dataset (55% in indoor and 59% in outdoor samples) could be explained by differences between

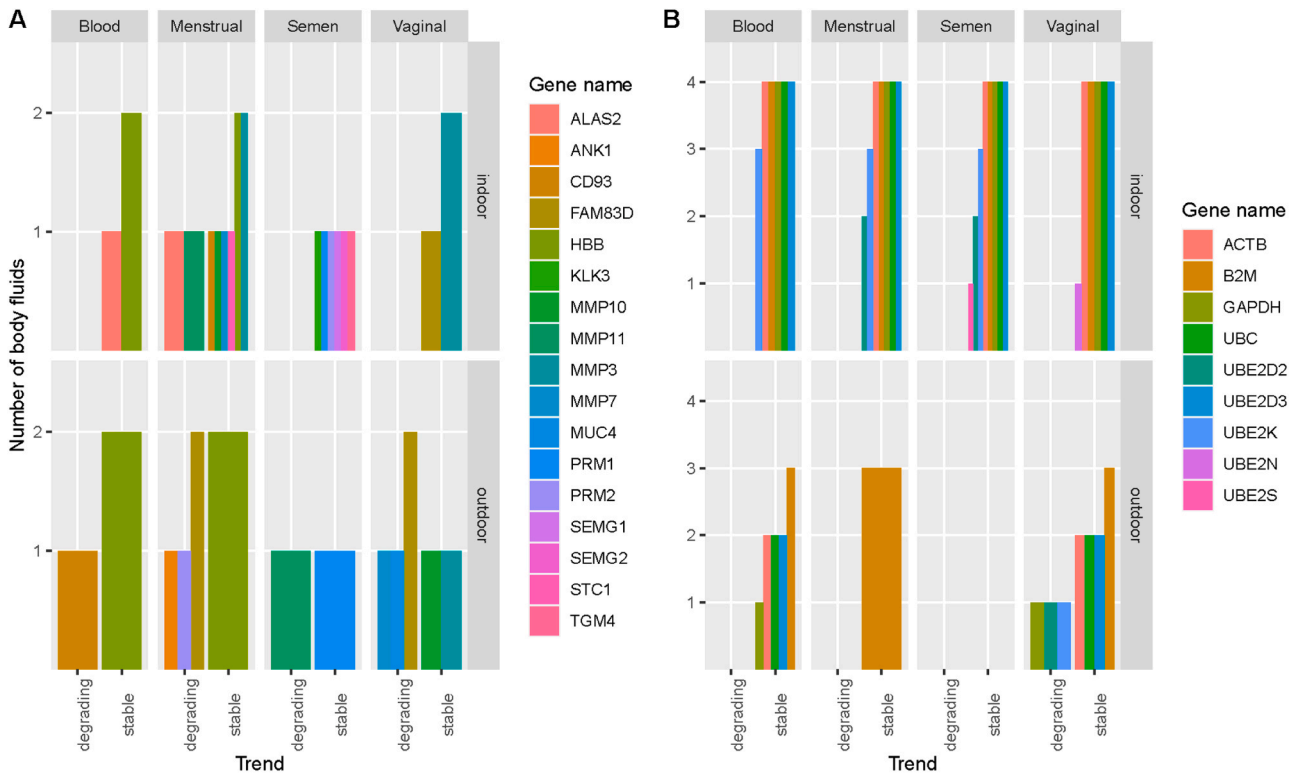

Fig. 5. Bar plots showing the commonality and uniqueness of body fluid-specific transcripts **A)** or housekeeping gene transcripts **B)**. A transcript can have a specific trend ("stable" or "degrading") in the indoor or outdoor samples exclusively in that body fluid (Number of body fluids =1 on the *y*-axis) or the trend can be visible in several body fluids. In the latter case, the transcript will have the bar height at 2 in two panels. *(For interpretation of the references to color in this figure, the reader is referred to the online version of this article.)*

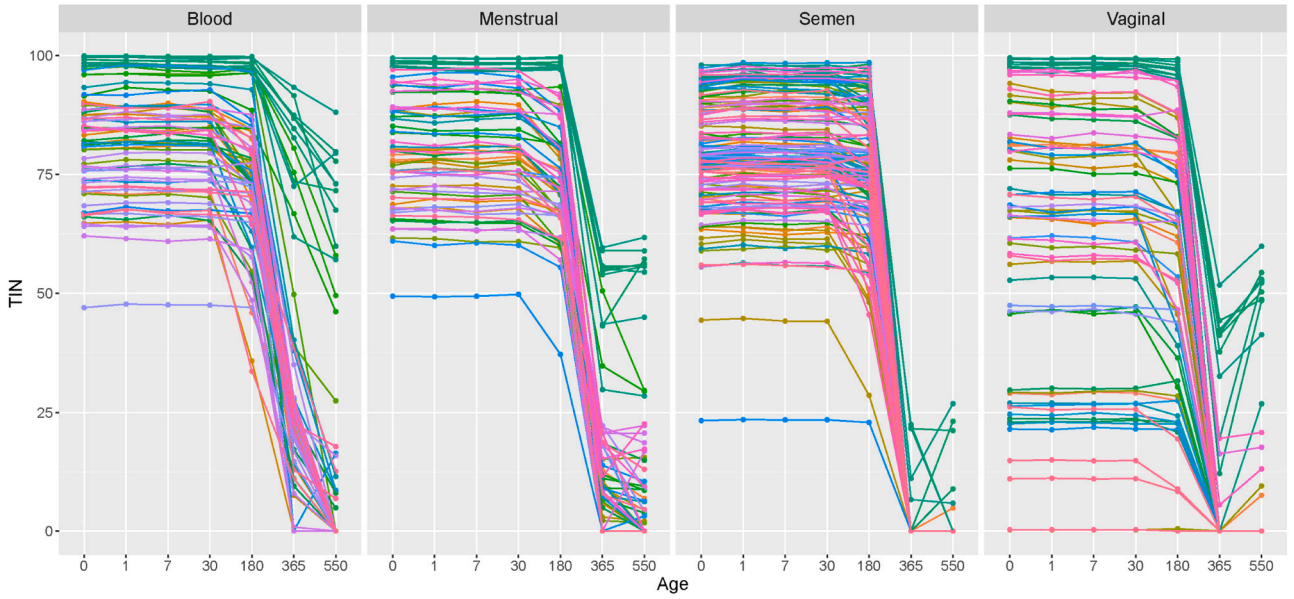

Fig. 6. TIN values of the indoor samples for the full time course of the transcripts fulfilling the "stable" criteria in the short term, up to 1 month (see Materials and Methods).

the body fluids (Fig. 1A and 1B). This confirms the findings from our previous study that we mainly perceive body fluid specific signatures [38]. However, we also detected a time effect based on gene expression accounting for about 25% of the variance (24% in indoor and 30% in outdoor samples). TIN-based NMDS analysis revealed a time effect in indoor samples separating time points up to 6 months from older samples, whereas no such separation was detected in outdoor samples (Fig. 1C and 1D). For saliva samples, a unique clustering pattern was observed in both conditions.

In depth analyses performed for each body fluid and storage condition revealed a drop in mRNA TIN values for indoor samples after one year, resulting in a high level of degradation (Fig. 2). In outdoor samples, we found a more fluctuating pattern with a sudden drop of mRNA TIN values at the 6 month time point. We realized that rRNA read counts

for the 6 months old samples were much higher than in samples previously analyzed using the same kit / lot for library preparation, indicating a bias for rRNA in sequencing results. Therefore, the issues with the 6 months old outdoor samples look like a technical rather than a biological phenomenon.

Saliva samples had very low human read counts. They not only showed a unique clustering pattern in the NMDS analyses, but exhibited also a distinct TIN distribution pattern, indicating complete degradation of the transcripts already shortly after deposition time. Therefore, we excluded saliva from further analyses. Weinbrecht et al. also excluded the saliva samples from data analysis due to a drastic decrease in human transcript abundance [37]. They reported that the vast majority of unaligned reads was of bacterial origin. This finding is not surprising, as the oral cavity is heavily populated by bacteria [48].

We assessed the degradation pattern of samples aged for up to 4 weeks in greater depth, for which more time points were analyzed per time interval. Focusing on the global degradation pattern, we found that the majority of transcripts do not show a clear trend (stable or degrading) but are, according to our specified criteria, fluctuating (referred to as "NA"). Indoor samples displayed a higher fraction of stable transcripts. This might be due to reduced RNase activity in dried stains [49]. In contrast, the outdoor samples showed more degrading transcripts. The placing of the samples on a rooftop made them accessible to environmental factors such as humidity, UV-light and wind, which are harmful to the RNA.

We then investigated whether specific characteristics (e.g. length or GC-content) of a transcript can be associated with a stable or degrading pattern. We found that genes with a lower GC-content were by trend more stable among the indoor samples and that shorter genes seemed to be more stable across multiple body fluids among the outdoor samples (Supplementary Figs. 1–4). In other words, the indoor environment (dark, room temperature) triggered a selection of transcripts based on GC-content, while the outdoor conditions (humidity, UV light, wind, etc.) selected transcripts based on length. A basic perception is that shorter transcripts are more stable than long ones [51]. Anderson et al. used the fact that short segments of the analyzed RNA species (β–actin and 18S-rRNA) are more stable than long ones for a TsD estimation approach [33]. However, Weinbrecht et al. did not find a relationship between transcript lengths and longevity in forensic body fluid stains in their transcriptome data [37]. Kudla et al. used HeLa and 293 T cells to investigate the relationship between GC-content and gene expression [52]. GC-rich genes were expressed up to a 100-fold more efficiently than their GC-poor counterparts. Besides, mRNA degradation rates were not correlated with GC content, suggesting that efficient transcription or mRNA processing is responsible for the high expression of GC-rich genes.

We realized that the experimental setup of the outdoor exposure might have had an impact on our outdoor results. Around 3 months after deposition of the samples on the rooftop, we observed dark spots on the semen swabs, which were placed in the front of the tray. The menstrual blood samples also displayed some dark spots and underwent a color change from dark red-brown to light red-brown. The blood, saliva and vaginal secretion samples showed less of the described changes, probably because they were better protected from wind etc. due to their placing in the backrows and their close proximity to a wall. The observed dark spots are most likely caused by accumulation of soil, pollen and dust. This pattern is also reflected in our results. After 4 weeks of exposure, only few stable genes common to indoor and outdoor samples of semen and menstrual blood could be identified, while blood and vaginal secretion showed an overlap of several hundred genes (Fig. 4). Semen and menstrual blood also showed many highly-to-completely degraded genes in outdoor samples, while blood and vaginal secretion were less affected (Supplementary Fig. 5). We conclude that the observed degradation pattern in the outdoor menstrual blood and semen samples might be due to the enhanced exposure to the environment. In addition, they are influenced by the local conditions: the location (rooftop of the institute building), the surrounding environment (park with trees and a forest in close proximity), the time of year, the outside temperature, etc.

Although not being the focus of our study, we could confirm that the majority of the known mRNA markers used for body fluid identification are quite stable when the samples are stored indoors and protected from light. Up to 6 months, the stability of genes in indoor samples was reasonably good, with the exception of menstrual blood showing signs of degradation already after 1 month (Fig. 5A and Supplementary Fig. 6). After 1 year, blood and menstrual blood genes were largely degraded, while semen and vaginal secretion genes were completely degraded.

Among the housekeeping genes, we found that 5 (*ACTB, B2M, GAPDH, UBC* and *UBE2D3*) out of the 10 candidates were stable across all body fluids in the indoor samples, whereas no gene was stable across all body fluids in outdoor samples (Fig. 5B). In the long term, the housekeeping genes showed a similar pattern as observed for the body fluid specific genes. They were either largely degraded (blood and menstrual blood) or completely degraded (semen and vaginal secretion) after 1 year (Supplementary Fig. 6). Several studies assessing TsD have investigated the degradation pattern of housekeeping genes in body fluid samples aged for weeks up to several months [30,32–35,37,50,53,54]. For *GAPDH*, ß-actin and *B2M* they report a degrading pattern [30,33–35,37,53,54]. For 18S-rRNA conflicting results were reported. Some studies identified 18S-rRNA to be relatively stable [30,32,33], while others reported a degrading pattern [31,34,35,54].

We performed an unbiased screening of the degradation patterns in order to identify putative markers showing a long term stability or a continuous degradation over time. Since no transcript with a significant resilience to degradation past the 1 year time point could be identified, the analyses were performed for time points up to 6 months. We were able to identify 24 stable markers for blood, 14 for menstrual blood, 69 for semen, 22 for vaginal secretion (Supplementary Table 3). Some overlap of detected markers was observed between body fluids (e.g. for ribosomal genes). rRNA has already been described by others to be stable [30,33]. Anderson and colleagues speculated that this might be due to the protective nature of associated proteins. Among the stable genes, we observed markers, which have been used for mRNA based body fluid identification (*HBB, HBA, SEMG1, SEMG2, PRM1*) highlighting their stable nature and suitability for body fluid identification [22–24]. In addition, we identified new stable transcripts in semen associated with the sperm cell, spermatogenesis or sperm motility. These could be evaluated as candidate markers for mRNA based semen identification. Besides, we also found 22 degrading genes for menstrual blood, 8 for semen, 23 for vaginal secretion, but none for blood (Supplementary Table 4).

A suitable approach for TsD estimation would be a targeted assay where an unstable transcript is analyzed in relation to a stable one, as previously proposed [30,33]. In an ideal case, an assay to determine the age of a stain would contain markers found in all body fluids so that no information about the stains composition is required. However, in our study, no transcript was identified to be stable or degrading across all body fluids and conditions assessed. Hence, this goal might be difficult to achieve. As already pointed out by Weinbrecht et al. it would be more reliable to base an assay on several instead of only a few transcripts [37]. A probabilistic or predictive approach might help finding suitable marker pairs [55–57]. Alternatively, in depth analysis of individual transcripts, as recently proposed by Fu et al., might be used to estimate the age of a stain [58]. They reported that in dried bloodstains the 5′-ends of selected mRNA transcripts degraded faster than the 3′-ends. The age of a bloodstain could be estimated with an accuracy of 2–4 weeks for stains up to 6 months old and an accuracy of 4–6 weeks for stains older than 6 months.

In summary, we found degradation signatures in forensically relevant body fluids in indoor and outdoor samples. The indoor samples showed a marked drop in RNA integrity after 6 months, while the outdoor samples were difficult to interpret. We detected several stable and

degrading transcripts in different body fluids in up to 6 months old indoor samples, which can be evaluated as candidate markers for TsD estimation. With our study, we could add to the existing knowledge about ex-vivo RNA degradation in forensically relevant body fluids. In a next step, we will analyze our data in more detail to see whether we are able to find degradation signatures at individual transcript levels. Besides, we intend to assess the degradation of other RNA species (e.g. miRNA, long-noncoding RNA). We will test the detected markers for their suitability as TsD estimators in a targeted assay.

Acknowledgements

This project was supported by the Swiss National Science Foundation (SNF, project-Nr. 310030L_175854). Special thanks go to Corinne Moser for technical assistance, Guro Dørum for help with interpretation of statistical data, and to all volunteers who provided samples for this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2021.102524.

References

[1] J.M. Butler, The future of forensic DNA analysis, Philos. Trans. R. Soc. Lond. B Biol. Sci. 370 (1674) (2015).
[2] C. Haas, E. Hanson, J. Ballantyne, mRNA and MicroRNA for body fluid identification, in: J. Siegel, P. Saukko (Eds.), Encyclopedia of Forensic Sciences, second ed., Academic Press, Waltham, 2013, pp. 402–408.
[3] J. Juusola, J. Ballantyne, mRNA profiling for body fluid identification by multiplex quantitative RT-PCR, J. Forensic Sci. 52 (6) (2007) 1252–1262.
[4] J. Juusola, J. Ballantyne, Multiplex mRNA profiling for the identification of body fluids, Forensic Sci. Int 152 (1) (2005) 1–12.
[5] J. Juusola, J. Ballantyne, Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification, Forensic Sci. Int 135 (2) (2003) 85–96.
[6] C. Haas, E. Hanson, J. Ballantyne, Capillary electrophoresis of a multiplex reverse transcription-polymerase chain reaction to target messenger RNA markers for body fluid identification, Methods Mol. Biol. 830 (2012) 169–183.
[7] C. Haas, B. Klesser, C. Maake, W. Bär, A. Kratzer, mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR, Forensic Sci. Int Genet. 3 (2) (2009) 80–88.
[8] C. Haas, et al., mRNA profiling for the identification of sperm and seminal plasma, Forensic Sci. Int. 2 (1) (2009) 534–535.
[9] C. Haas, E. Hanson, A. Kratzer, W. Bär, J. Ballantyne, Selection of highly specific and sensitive mRNA biomarkers for the identification of blood, Forensic Sci. Int. Genet. 5 (5) (2011) 449–458.
[10] C. Haas, E. Hanson, W. Bär, R. Banemann, A.M. Bento, A. Berti, E. Borges, C. Bouakaze, A. Carracedo, M. Carvalho, A. Choma, M. Dötsch, M. Durianciková, P. Hoff-Olsen, C. Hohoff, P. Johansen, P.A. Lindenbergh, B. Loddenkötter, B. Ludes, O. Maroñas, N. Morling, H. Niederstätter, W. Parson, G. Patel, C. Popielarz, E. Salata, P.M. Schneider, T. Sijen, B. Sviezená, L. Zatkalíková, J. Ballantyne, mRNA profiling for the identification of blood–results of a collaborative EDNAP exercise, Forensic Sci. Int. Genet. 5 (1) (2011) 21–26.
[11] C. Haas, E. Hanson, M.J. Anjos, R. Banemann, A. Berti, E. Borges, A. Carracedo, M. Carvalho, C. Courts, G. De Cock, M. Dötsch, S. Flynn, I. Gomes, C. Hollard, B. Hjort, P. Hoff-Olsen, K. Hríbiková, A. Lindenbergh, B. Ludes, O. Maroñas, N. McCallum, D. Moore, N. Morling, H. Niederstätter, F. Noel, W. Parson, C. Popielarz, C. Rapone, A.D. Roeder, Y. Ruiz, E. Sauer, P.M. Schneider, T. Sijen, D. S. Court, B. Sviežená, M. Turanská, A. Vidaki, L. Zatkalíková, J. Ballantyne, RNA/DNA co-analysis from human saliva and semen stains–results of a third collaborative EDNAP exercise, Forensic Sci. Int. Genet. 7 (2) (2013) 230–239.
[12] C. Haas, E. Hanson, M.J. Anjos, W. Bär, R. Banemann, A. Berti, E. Borges, C. Bouakaze, A. Carracedo, M. Carvalho, V. Castella, A. Choma, G. De Cock, M. Dötsch, P. Hoff-Olsen, P. Johansen, F. Kohlmeier, P.A. Lindenbergh, B. Ludes, O. Maroñas, D. Moore, M.L. Morerod, N. Morling, H. Niederstätter, F. Noel, W. Parson, G. Patel, C. Popielarz, E. Salata, P.M. Schneider, T. Sijen, B. Sviežena, M. Turanská, L. Zatkalíková, J. Ballantyne, RNA/DNA co-analysis from blood stains–results of a second collaborative EDNAP exercise, Forensic Sci. Int. Genet. 6 (1) (2012) 70–80.
[13] C. Haas, E. Hanson, M.J. Anjos, K.N. Ballantyne, R. Banemann, B. Bhoelai, E. Borges, M. Carvalho, C. Courts, G. De Cock, K. Drobnic, M. Dötsch, R. Fleming, C. Franchi, I. Gomes, G. Hadzic, S.A. Harbison, J. Harteveld, B. Hjort, C. Hollard, P. Hoff-Olsen, C. Hüls, C. Keyser, O. Maroñas, N. McCallum, D. Moore, N. Morling, H. Niederstätter, F. Noël, W. Parson, C. Phillips, C. Popielarz, A.D. Roeder, L. Salvaderi, E. Sauer, P.M. Schneider, G. Shanthan, D.S. Court, M. Turanská, R. A. van Oorschot, M. Vennemann, A. Vidaki, L. Zatkalíková, J. Ballantyne, RNA/DNA co-analysis from human menstrual blood and vaginal secretion stains: results of a fourth and fifth collaborative EDNAP exercise, Forensic Sci. Int. Genet. 8 (1) (2014) 203–212.
[14] C. Haas, E. Hanson, R. Banemann, A.M. Bento, A. Berti, Á. Carracedo, C. Courts, G. Cock, K. Drobnic, R. Fleming, C. Franchi, I. Gomes, G. Hadzic, S.A. Harbison, B. Hjort, C. Hollard, P. Hoff-Olsen, C. Keyser, A. Kondili, O. Maroñas, N. McCallum, P. Miniati, N. Morling, H. Niederstätter, F. Noël, W. Parson, M.J. Porto, A. D. Roeder, E. Sauer, P.M. Schneider, G. Shanthan, T. Sijen, D. Syndercombe Court, M. Turanská, M. van den Berge, M. Vennemann, A. Vidaki, L. Zatkalíková, J. Ballantyne, RNA/DNA co-analysis from human skin and contact traces–results of a sixth collaborative EDNAP exercise, Forensic Sci. Int. Genet. 16 (2015) 139–147.
[15] E.K. Hanson, J. Ballantyne, Highly specific mRNA biomarkers for the identification of vaginal secretions in sexual assault investigations, Sci. Justice 53 (1) (2013) 14–22.
[16] E. Hanson, et al., mRNA MPS tissue identification assay to aid in the investigation of traumatic injuries, Forensic Sci. Int. 7 (1) (2019) 25–26.
[17] I. Gomes, F. Kohlmeier, P. Schneider, Genetic markers for body fluid and tissue identification in forensics, Forensic Sci. Int. 3 (1) (2011) e469–e470.
[18] A. Lindenbergh, M. van den Berge, R.J. Oostra, C. Cleypool, A. Bruggink, A. Kloosterman, T. Sijen, Development of a mRNA profiling multiplex for the inference of organ tissues, Int. J. Leg. Med. 127 (5) (2013) 891–900.
[19] E. Hanson, J. Ballantyne, Human organ tissue identification by targeted RNA deep sequencing to aid the investigation of traumatic injury, Genes 8 (11) (2017).
[20] S. Ingold, G. Dørum, E. Hanson, A. Berti, W. Branicki, P. Brito, P. Elsmore, K. B. Gettings, F. Giangasparo, T.E. Gross, S. Hansen, E.N. Hanssen, M.L. Kampmann, M. Kayser, F.X. Laurent, N. Morling, A. Mosquera-Miguel, W. Parson, C. Phillips, M. J. Porto, E. Pośpiech, A.D. Roeder, P.M. Schneider, K. Schulze Johann, C.R. Steffen, D. Syndercombe-Court, M. Trautmann, M. van den Berge, K.J. van der Gaag, J. Vannier, V. Verdoliva, A. Vidaki, C. Xavier, J. Ballantyne, C. Haas, Body fluid identification using a targeted mRNA massively parallel sequencing approach – results of a EUROFORGEN/EDNAP collaborative exercise, Forensic Sci. Int. Genet. 34 (2018) 105–115.
[21] M. van den Berge, T. Sijen, Extended specificity studies of mRNA assays used to infer human organ tissues and body fluids, Electrophoresis 38 (24) (2017) 3155–3160.
[22] F. Kohlmeier, P.M. Schneider, Successful mRNA profiling of 23 years old blood stains, Forensic Sci. Int. Genet. 6 (2) (2012) 274–276.
[23] D. Zubakov, M. Kokshoorn, A. Kloosterman, M. Kayser, New markers for old stains: stable mRNA markers for blood and saliva identification from up to 16-year-old stains, Int. J. Leg. Med. 123 (1) (2009) 71–74.
[24] M. Sirker, P.M. Schneider, I. Gomes, A 17-month time course study of human RNA and DNA degradation in body fluids under dry and humid environmental conditions, Int. J. Leg. Med. 130 (6) (2016) 1431–1438.
[25] R.H. Bremmer, K.G. de Bruin, M.J. van Gemert, T.G. van Leeuwen, M.C. Aalders, Forensic quest for age determination of bloodstains, Forensic Sci. Int. 216 (1–3) (2012) 1–11.
[26] E.K. Hanson, J. Ballantyne, A blue spectral shift of the hemoglobin soret band correlates with the age (time since deposition) of dried bloodstains, PLoS One 5 (9) (2010) 12830.
[27] K.C. Doty, G. McLaughlin, I.K. Lednev, A Raman "spectroscopic clock" for bloodstain age determination: the first week after deposition, Anal. Bioanal. Chem. 408 (15) (2016) 3993–4001.
[28] K.C. Doty, C.K. Muro, I.K. Lednev, Predicting the time of the crime: bloodstain aging estimation for up to two years, Forensic Chem. 5 (2017) 1–7.
[29] M. Bauer, S. Polzin, D. Patzelt, Quantification of RNA degradation by semi-quantitative duplex and competitive RT-PCR: a possible indicator of the age of bloodstains? Forensic Sci. Int. 138 (1–3) (2003) 94–103.
[30] S. Anderson, B. Howard, G.R. Hobbs, C.P. Bishop, A method for determining the age of a bloodstain, Forensic Sci. Int. 148 (1) (2005) 37–45.
[31] C. Hampson, J. Louhelainen, S. McColl, An RNA expression method for aging forensic hair samples, J. Forensic Sci. 56 (2) (2011) 359–365.
[32] B. Qi, L. Kong, Y. Lu, Gender-related difference in bloodstain RNA ratio stored under uncontrolled room conditions for 28 days, J. Forensic Leg. Med. 20 (4) (2013) 321–325.
[33] S.E. Anderson, G.R. Hobbs, C.P. Bishop, Multivariate analysis for estimating the age of a bloodstain, J. Forensic Sci. 56 (1) (2011) 186–193.
[34] S. Alshehhi, P.R. Haddrill, Estimating time since deposition using quantification of RNA degradation in body fluid-specific markers, Forensic Sci. Int. 298 (2019) 58–63.
[35] S. Alshehhi, N.A. McCallum, P.R. Haddrill, Quantification of RNA degradation of blood-specific markers to indicate the age of bloodstains, Forensic Sci. Int. 6 (2017) e453–e455.
[36] M.H. Lin, D.F. Jones, R. Fleming, Transcriptomic analysis of degraded forensic body fluids, Forensic Sci. Int. Genet. 17 (2015) 35–42.
[37] K.D. Weinbrecht, J. Fu, M. Payton, R. Allen, Time-dependent loss of mRNA transcripts from forensic stains, Res. Rep. Forensic Med. Sci. 7 (2017) 1–12.
[38] A.P. Salzmann, et al., Transcription and microbial profiling of body fluids using a massively parallel sequencing approach, Forensic Sci Int Genet 43 (2019), 102149.
[39] Tecan Genomics. *Trio RNA-Seq – Product Sheet.* 2019 [cited 2020 10 14]; Available from: ⟨https://www.nugen.com/sites/default/files/401141_v1_Product_Sheet%3A_Trio_RNA-Seq_4145.pdf⟩.
[40] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data, Bioinformatics 30 (15) (2014) 2114–2120.
[41] E. Kopylova, L. Noe, H. Touzet, SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data, Bioinformatics 28 (24) (2012) 3211–3217.

[42] A. Dobin, C.A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, T.R. Gingeras, STAR: ultrafast universal RNA-seq aligner, Bioinformatics 29 (1) (2013) 15–21.
[43] M. Lawrence, W. Huber, H. Pagès, P. Aboyoun, M. Carlson, R. Gentleman, M. T. Morgan, V.J. Carey, Software for computing and annotating genomic ranges, PLoS Comput. Biol. 9 (8) (2013), 1003118.
[44] M.D. Robinson, D.J. McCarthy, G.K. Smyth, edgeR: a Bioconductor package for differential expression analysis of digital gene expression data, Bioinformatics 26 (1) (2010) 139–140.
[45] L. Wang, J. Nie, H. Sicotte, Y. Li, J.E. Eckel-Passow, S. Dasari, P.T. Vedell, P. Barman, L. Wang, R. Weinshiboum, J. Jen, H. Huang, M. Kohli, J.P. Kocher, Measure transcript integrity using RNA-seq data, BMC Bioinforma. 17 (1) (2016) 58.
[48] J.L. Pope, S. Tomkovich, C. Jobin, Chapter 9 – influence of Commensal Microbiota and Metabolite for Mucosal Immunity, in: H. Kiyono, D.W. Pascual (Eds.), Mucosal Vaccines, Second edition.,, Academic Press, 2020, pp. 143–164.
[49] S.L. Fordyce, M.L. Kampmann, N.L. van Doorn, M.T. Gilbert, Long-term RNA persistence in postmortem contexts, Investig. Genet. 4 (1) (2013) 7.
[50] M. Bauer, D. Patzelt, A method for simultaneous RNA and DNA isolation from dried blood and semen stains, Forensic Sci. Int. 136 (1–3) (2003) 76–78.
[51] L. Feng, D.-K. Niu, Relationship between mRNA stability and length: an old question with a new twist, Biochem. Genet. 45 (1) (2007) 131–137.
[52] G. Kudla, L. Lipinski, F. Caffin, A. Helwak, M. Zylicz, High guanine and cytosine content increases mRNA levels in mammalian cells, PLoS Biol. 4 (6) (2006) 180.
[53] T.A.G. Bird, L. Walton-Williams, G. Williams, Time since deposition of biological fluids using RNA degradation, Forensic Sci. Int. 7 (1) (2019) 401–402.
[54] A.-M. Simard, L. DesGroseillers, V. Sarafian, Assessment of RNA stability for age determination of body fluid stains, Can. Soc. Forensic Sci. J. 45 (4) (2012) 179–194.
[55] G. Dørum, S. Ingold, E. Hanson, J. Ballantyne, G. Russo, S. Aluri, L. Snipen, C. Haas, Predicting the origin of stains from whole miRNome massively parallel sequencing data, Forensic Sci. Int. Genet. 40 (2019) 131–139.
[56] G. Dørum, S. Ingold, E. Hanson, J. Ballantyne, L. Snipen, C. Haas, Predicting the origin of stains from next generation sequencing mRNA data, Forensic Sci. Int. Genet. 34 (2018) 37–48.
[57] D. Iacob, A. Fürst, T. Hadrys, A machine learning model to predict the origin of forensically relevant body fluids, Forensic Sci. Int. 7 (1) (2019) 392–394.
[58] J. Fu, R.W. Allen, A method to estimate the age of bloodstains using quantitative PCR, Forensic Sci. Int. Genet. 39 (2019) 103–108.